IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 5291
Carcagno *et al.*	Art Unit: 1654
Appl. No. 09/830,964	Examiner: Leith, Patricia A.
§ 371 Date: November 5, 2001	Atty. Docket: 1909.0030002/JAG/PAJ
For: **Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants**	

Declaration Under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, Dr. Marcelo E. Criscuolo, residing at Guevara 349, Buenos Aires, Argentina, declare and state as follows:

1. I am an employee of Bio Sidus S.A. The owner of the patent application is Sterrenbeld Biotechnologie North America, Inc., which company is wholly owned by Bio Sidus S.A. I am also a co-inventor on the patent application.

2. An accurate list of my credentials is set forth in my *Curriculum Vitae* attached as Appendix 1.

3. I have reviewed the above-captioned patent application and the Office Action dated June 1, 2004. I have also reviewed the claims of the captioned patent application.

4. The patent application discloses at pages 7-9 several methods of purifying human erythropoietin according to the invention. Experiments directed to erythropoietin purification were also disclosed on pages 9-16 using one set of purification steps. As shown in Appendix 2, an alternative erythropoietin purification process as discussed in the captioned application was tested under my direction, resulting in the successful purification of human erythropoietin.

5. An additional alternative purification processes of purifying recombinant

human erythropoietin from cell supernatants was employed, comprising a combination of the steps disclosed in the patent application (differential saline precipitation; hydrophobic interaction chromatography; two concentration and diafiltration steps; anionic exchange chromatography; cationic exchange chromatography; and molecular exclusion chromatography), but in a different order. The attached data provide a description of the alternative process.

6. Given that the protein purification protocol containing the steps described in the specification results in a purified erythropoietin, and that these steps may be employed in varying order, it is my opinion that one of ordinary skill in the art would expect to successfully purify erythropoietin using the methods of the present invention. On information and belief, one of ordinary skill would also know how to vary the steps of the purification protocol to obtain purified erythropoietin through the claimed method.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,

Dr. Marcelo E. Criscuolo

Date: 12/01/04

338810.1

CURRICULUM VITAE
2004

NAME	Marcelo Eduardo Criscuolo
DATE AND PLACE OF BIRTH	February 14, 1953, Buenos Aires
ID	10.822.775
MARITAL STATUS	Divorced, two children
ADDRESS	Guevara 349, C.A.B.A.
E-MAIL ADDRESS	mec@biosidus.com.ar

EDUCATION

1976 Graduate School of Pharmacy and Biochemistry, University of Buenos Aires.
Degree: Biochemist.

PROFESSIONAL BACKGROUND

1990 - 2003 Bio Sidus S.A. Position: Executive Director

1989 - 1990 Bio Sidus S.A. Position: Scientific-Technical Coordinator

1981 - 1990 Bio Sidus S.A. Position: Head Protein Department

1980 - 1981 Chemar S.A. Position: Advisor for the development of diagnostic kits.

1979 - 1980 Inmunoquemia S.A. Position: Professional in charge of Protein Development

1976 - 1979 Laboratory of Vasoactive Substances
Institute of Medical Investigation directed by Dr. Víctor E. Nahmod.

1977 - 1991 Direction and management of clinical laboratories in private hospitals.

TEACHING ACTIVITY

1975 - 1977 Professor at the Department of Physicochemistry, School of Pharmacy and Biochemistry, University of Buenos Aires.

FELLOWSHIPS

1986 Institute of Immunology, Luminy, Marseilles, France
Directed by Dr. Goldstein
Subject: 2-5 Oligo A synthetase system

1983 Department of Virology, Institut Curie, Paris, France.
Directed by Dr. Ernesto Falcoff
Subject: Gamma interferon

1977 Fellow of the Scientific Committee of Research, Argentina

COLLABORATIONS IN THE FIELD OF BIOTECHNOLOGY

- Direct participation as officer in charge of the Protein Department of Bio Sidus S.A. in the development and scaling up of *upstream* and *downstream* processes of the following recombinant human proteins, currently distributed in over thirty countries

  a. **Erythropoietin** – Launched in Argentina in 1990
  b. **Interferon alfa 2b** - Launched in Argentina in 1991
  c. **Filgrastim** - Launched in Argentina in 1995
  d. **Somatropin** - Launched in Argentina in 1997
  e. **Lenograstim** - Launched in Argentina in 1998
  f. **Interferon alfa 2a** - Launched in Argentina in 1998

- In collaboration with renowned international centers, development of controlled release systems for biomolecules.

- Direction of the "Pharmaceutical Dairy Project". *Project description*: development of cloned transgenic animals to obtain therapeutic molecules in milk of mammals. First stage of the project concluded with the birth of the first cloned calf in Latin America (Pampa) in August 2002. Second stage of the project in course: birth of cloned calves transgenic for the human growth hormone gene.

- In collaboration with CERELA and PROIMI (Government Research Centers), development of a probiotic medicine to treat gastrointestinal disorders. Marketed in Argentina since 2002.

- Direction of the project: "Gene Therapy Directed to the Angiogenesis of Ischemic Tissues", developed by Bio Sidus in collaboration with Fundación Favaloro. *Project description:* development of a pharmaceutical product having as active ingredient a plasmid carrying the Vascular Endothelial Growth Factor (VEGF) gene. Injection of this plasmid might replace current surgical options such as coronary by-pass or cardiac graft, offering a simpler and safer therapeutic alternative. Patients with peripheral ischemic episodes will also benefit from this therapy since it could prevent amputation of damaged limbs. Pre-clinical assays on pigs and goats showed promising results. Protocols for Phase I clinical trials have been submitted to the ethics committees of Argentinean Regulatory Authorities.

- Direction of the project "Gene Therapy directed to Cancer Treatment", in collaboration with the Instituto Roffo, Argentina. *Project description*: Construction of plasmids carrying the gene codifying for the Herpes Virus Timidine Kinase (TK). Treatment has been designed through the injection of plasmid into tumor cells to induce production of TK plus simultaneous administration of Gancyclovir to induce

destruction of tumor cells. Ongoing pre-clinical trials in dogs with melanoma.

- Direction of plant biotechnology projects:

  a. Obtention of several events of potatoes resistant to viral pathogens (PVY virus) through incorporation of a viral gene in the plant genome. The result of this genetic modification is the inhibition of viral replication in the cells of plant individuals that could correctly incorporate said transgene. Two transgenic varieties have been obtained which have been registered at the Direction of Variety Registration of the Secretariat of Agriculture, Livestock, Fishing and Food.

  b. Strategy described in "a" is being applied to other local plant varieties (sugar cane) with promising results.

  c. Obtention of several potato events resistant to herbicides (Glyfosinate).

  d. Development of a genotypification system for grape strains, project in collaboration with the Argentinean Institute of Vitiviniculture, to genetically certify grape varieties.

  e. Development of a system to produce biomolecules in tobacco plants (*Molecular farming*).

  f. Development of micropropagation techniques (specially *berries*).

PUBLICATIONS

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J, Landi D, Rendo P, Sánzhez Avalos JC, Criscuolo ME, Díaz A.
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Investigación de anticuerpos en pacientes tratados con interferon alfa 2 recombinante.
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Tratamiento con eritropoyetina recombinante humana, rHuEPO, en pacientes prematuros, de 34S, de muy bajo peso.
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Criscuolo ME, Vidal A, Mella D, Caputo M, Díaz A.
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Criscuolo ME, Mella D, Caputo M, Díaz A.

SAIC Mar del Plata, Argentina, 1982

DR. MARCELO EDUARDO CRISCUOLO
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Biochemist graduated at the School of Pharmacy and Biochemistry of the University of Buenos Aires. Fellow of the Scientific Research Commission and Researcher at the Laboratory of Vasoactive Substances of the Institute of Medical Investigation of the University of Buenos Aires. Teaching experience in the Department of Physicochemistry of the School of Pharmacy and Biochemistry, University of Buenos Aires, awarded in 1975 with the Prize Incentive for Scientific Research granted by said University. Invited in 1983 as fellow by the Institute Curie, Paris, to conduct research on the subject "Gamma Interferon" at the Department of Virology directed by Prof. Ernesto Falcoff. In 1986, in the framework of a French-Argentinean collaborative program for the development of sciences, invited by the CTBio to conduct research on the "2' 5' - oligoA synthetase system"as fellow at the Institute of Immunology, Luminy, Marseilles, under direction of Dr. Goldstein.

Professional activity in the biotech industry started in 1981 at Bio Sidus, formerly as Head of the Area of Protein Purification, then as Scientific-Technical Coordinator and since 1990, as Executive Director.

The professional activities in the field of biotechnology have been acknowledged through a series of personal awards which include the Health 2000 Prize awarded by the Ministry of Health of Argentina, and the Technological Leader Award granted by FUNPRECIT. Recently awarded with the Platinum Konex Prize 2003, in the area of Biotechnology.

Representative for Argentina of the *International Society for Interferon and Cytokine Research (ISICR)*, member of the National Committee of Biotechnology and Health, currently drafting the guidelines for registration and control of biotechnological medicines. Contributed to the preparation of the National Pharmacopoeia, Biotech section.

Appendix 2

Alternative Purification Process

Recovery (a)

7,920 g of ammonium sulfate were dissolved in 30 liters of sterile concentrated solution obtained from culturing CHO (Chinese Hamster Ovary) cells producing EPO. After addition of ammonium sulfate, the solution was stored at 4 °C for 24 hours. Many contaminant proteins precipitated while the EPO remained in solution. The product was centrifuged at 5,000 RPM for 10 minutes in a Sorvall centrifuge, using a HG4L rotor.

Concentration and Diafiltration (c)

The material obtained from the previous step is concentrated and diafiltered according to the conditions described below:

1. Equipment:
   A. Peristaltic pump: Watson Marlow - Cat. N° 302S
   B. Tubing: Masterflex - Cat. N° 06402-18
   C. Concentrator: Prep Scale Millipore CDU F006LC

2. Solutions and buffers:
   A. 10 mM Sodium Dodecyl Sulfate (SDS)
   B. 1 mM Triton X-100
   C. 0.1 N NaOH
   D. Buffer A: 10 mM NaH_2PO_4, pH 7.2

3. Material to be processed:
   A. Selected fractions resulting from the previous example.
   B. Sample conditions:
      1) Volume: 30,000 ml
      2) pH: 7.2

The equipment was first cleaned, sanitized and equilibrated, and the following sequence of solutions and buffers were flowed through the equipment: 10 l of 10 mM SDS; 40 l of H_2O; 10 l of 1 mM Triton X-100, 40 l of H_2O; 10 l of 0.1 N NaOH; 40 l of H_2O; and, finally, 5 l of Buffer A. The equipment was then ready to be used for concentration and diafiltration against Buffer A on the selected fractions, following the usual methodology.

The final volume of the concentrated product was between 2,000 to 3,000 ml, its conductivity was 1,100-1,550 μS/cm, and its pH, 7.2.

Anionic Exchange Chromatography (d)

The material resulting from the previous example was chromatographed using an anionic exchange matrix, as follows:

1. Equipment:
   A. Column:
      1) Diameter: 5.6 cm
      2) Bed height: 41 cm
      3) Matrix
         a. Q-Sepharose Fast Flow (Pharmacia)
         b. Volume: 1 l

2. Solutions and buffers:
   A. Buffer A: 10mM NaH_2PO_4, pH 7.2
   B. Buffer H: 50mM NaH_2PO_4, pH 7.2
   C. Buffer C: 200 mM NaH_2PO_4, 500 mM NaCl, pH 7.2
   D. 0.5 N NaOH

3. Material to be chromatographed
   A. Ammonium sulfate supernatant, duly concentrated and diafiltered.
   B. Sample conditions:
      1) Volume: 2,000 to 3,000 ml
      2) Conductivity: 1,100-1,550 μS/cm
      3) pH: 7.2

To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 volume of the column ("vc") (1 l) of H_2O; 1.0 vc (1 l) of 0.5 N NaOH; 2.0 vc (2 l) of H_20; 1.0 vc (1 l) of Buffer H; 1.0 vc (1 l) of Buffer A; 1.5 vc (1.5 l) of Buffer C; and, finally, 5.0 vc (5 l) of Buffer A.

Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at room temperature at 30 ± 5 cm/hour. Thereafter, the elution was performed at the same flow rate and temperature, and the solutions and buffers hereinafter detailed were passed in the following order: 1.5 vc (1.5 l) of Buffer A; thereafter, a gradient of Buffer A-Buffer C was applied, starting from a 100:0 ratio of said buffers until a 86:14 ratio of said buffers in a total volume of 2.0 vc (2 l) was reached; once the gradient had finished, a Buffer A-Buffer C step (86:14) in a total volume of 2.0 vc (2 l) was performed; thereafter, a gradient of Buffer A-Buffer C was applied, starting from a 86:14 ratio of said buffers until a 67:33 ratio of said buffers in a total volume of 1.0 vc (1 l) was reached; once the gradient had finished, a Buffer A-Buffer C step (67:33) in a total volume of 2.0 vc (2 l) was performed; once the step had finished, 1.5 vc (1.5 l) of Buffer C were passed through the column. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4 °C.

Cationic Exchange Chromatography (e)

The material resulting from the previous example was chromatographed using a cationic exchange matrix, as follows:

1. Equipment:
   - A. Column:
     1) Diameter: 5.6 cm
     2) Bed height: 41 cm
     3) Matrix
        a. SP-Sepharose Fast Flow (Pharmacia)
        b. Volume: 1 l

2. Solutions and buffers
   - A. Buffer D: 12.5 mM $Na_2HPO_4.12H_2O$, 4 mM Citric Acid, pH 6.0

B. Buffer E: 12.5 mM $Na_2HPO_4.12H_2O$, 4 mM Citric Acid, 500 mM NaCl, pH 6.0

C. 0.5 N NaOH

3. Material to be chromatographed

A. Fractions selected from the previous example, adjusted to pH 6.0 ± 0.1 with solid $Na_2HPO_4.12H_2O$ and diluted until reaching a conductivity of 5.5 ± 0.4 mS/cm.

B. Sample conditions:

1) Volume: 3,000 ± 500 ml
2) Conductivity: 5.5 ± 0.4 mS/cm
3) pH: 6.0 ± 0.1

To equilibrate the column, the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (1 l) of H_2O; 1.0 vc (1 l) of 0.5 N NaOH; 1.0 vc (1 l) of Buffer E; and, finally, 1.5 vc (1.5 l) of Buffer D.

Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at room temperature at 30 ± 5 cm/hour. Thereafter, the elution was performed at the same flow rate and temperature, and the solutions and buffers hereinafter detailed were passed through it in the following order: 1.5 vc (1.5 l) of Buffer D; thereafter, a gradient of Buffer D-Buffer E was applied starting from a 100:0 ratio of said buffers until a 50:50 ratio of said buffers in a total volume of 2.0 vc (2 l) was reached; once the gradient had finished, 1.0 vc (1 l) of Buffer E was passed through the column. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4 °C.

Hydrophobic Interaction Chromatography (b)

The material obtained from the previous step, properly conditioned, is chromatographed using an Hydrophobic Interaction matrix (Phenyl Sepharose 6 Fast Flow low sub.-Pharmacia) according to the following parameters:

1. Equipment:

A. Column:

1) Diameter: 5.6 cm

2) Bed height: 20 cm

3) Matrix:

a. Phenyl-Sepharose 6 Fast Flow low sub. (Pharmacia)

b. Volume: 500 ml

2. Solutions and buffers:

A. Buffer A: 10mM NaH_2PO_4, pH 7.2

B. Buffer F: 10mM NaH_2PO_4, 1.8 M $(NH_4)_2SO_4$, pH 7.2

C. 20% isopropyl alcohol

D. 0.5 N NaOH

3. Material to be chromatographed:

A. Selected fractions from previous example.

B. Sample conditions:

1) Volume: 6,000 ml

2) Conductivity: 5,000-8,000 μS/cm

3) pH: 7.2

To equilibrate and sanitize the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (500 ml) of H_2O; 1.0 vc (500 ml) of 20% isopropyl alcohol; 1.0 vc (500 ml) of H_2O; 1.0 vc (500 ml) of NaOH 0.5 N; 1.0 vc (500 ml) of H_2O; and, finally, 1.0 vc (500 ml) of Buffer F.

Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at 4 °C, at a 19 cm/hour flow. Thereafter, the elution was performed at the same flow rate but at room temperature, and the solutions and buffers hereinafter detailed were passed through the column in the following quantities and order: 1.0 vc (500 ml) of Buffer F; thereafter, a gradient of Buffer F-Buffer A was applied, starting from a 85:15 ratio of said buffers until 50:50 ratio of said buffers in a total volume of 10.0 vc (5,000 ml) was reached; when the gradient had finished, 1.0 vc (500 ml) of Buffer F-Buffer A in a 30:70 ratio was passed through the column; thereafter, 1.0 vc (500 ml) of H_2O, and, finally, 1.0 vc (500 ml) of 20 % isopropyl alcohol were passed through the column. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

Concentration and Diafiltration (f)

The fractions resulting from the previous example were concentrated and diafiltered according to the following parameters and conditions:

1. Equipment:
   A. Peristaltic pump: Watson Marlow - Cat. N° 302S
   B. Tubing: Masterflex - Cat. N° 06402-18
   C. Concentrator: Prep Scale Millipore CDU F002LC

2. Solutions and buffers:
   A. 10 mM Sodium Dodecyl Sulfate (SDS)
   B. 1 mM Triton X-100
   C. 0.1 N NaOH
   D. Buffer B: 10 mM NaH_2PO_4, 150 mM NaCl, 0.05 mg/ml Lactose, pH 7.2

3. Material to be processed:
   A. Selected fractions resulting from the previous step.
   B. Sample conditions:
      1) Volume: 3,000-6,000 ml
      2) Conductivity: 150 ± 30 mS/cm
      3) pH: 6.0

The equipment was first, cleaned, sanitized and equilibrated, letting pass through it the following sequence of solutions and buffers: 10 l of 10 mM SDS; 40 l of H_2O; 10 l of 1 mM Triton X-100, 40 l of H_2O; 10 l of 0.1 N NaOH; 40 l of H_2O; and, finally, 5 l of Buffer B. In this way, the equipment was ready to be used for the concentration and diafiltration procedures against Buffer B on the selected fractions, following the usual methodology.

The conductivity and pH of the final product were 15,500-19,000 mS/cm and 7.2, respectively. The solution was stored at 4 °C.

Molecular Exclusion Chromatography (g)

The material resulting from the previous example was chromatographed using a molecular exclusion matrix, as follows:

1. Equipment:
   A. Column:
      1) Diameter: 5.6 cm
      2) Bed height: 73 cm
      3) Matrix
         a. Sephacryl S-200 HP (Pharmacia)
         b. Volume: 1,800 ml

2. Solutions and buffers:
      1) Buffer B: 10mM NaH_2PO_4, 150 mM NaCl, 0.05 mg/ml Lactose, pH 7.2
      2) 0.5 N NaOH

Material to be chromatographed

A. Fractions selected from Example 13, concentrated and diafiltered.
B. Sample conditions:
   1) Volume: 350 to 600 ml
   2) Conductivity: 15,500-19,000 mS/cm
   3) pH: 7.2

To equilibrate the column, the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (1,800 ml) of H_2O; 1.0 vc (1,800 ml) of 0.5 N NaOH; 1.0 vc (1,800 ml) of H_2O; and, finally, 3.0 vc (5,400 ml) of Buffer B. Once the column was equilibrated, 34 ml from the material to be chromatographed were loaded. Said loading was performed at room temperature at 25 ± 3 cm/hour. Thereafter, the elution was performed at the same flow and temperature rates, and 0.75 vc (1,350 ml) of Buffer B were passed through the column. This procedure was repeated until the material to be chromatographed was completely utilized. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4 °C.

With this step, the purification process was concluded, and the EPO obtained

by means of it was assayed for identity and biological activity as stated in Example 8.

The global yield of the entire purification process and the purity degree of the EPO obtained by means of this process were very similar to those stated in Example 7 of the application.